Supplementary Information for

Toward Photoswitchable Electronic Pre-Resonance Stimulated Raman Probes

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EXPERIMENTAL METHODS

Stimulated Raman Scattering Microscopy. A picoEMERALD (Applied Physics and Electronics) system provided the pump (770–990 nm wavelength, 2 ps pulse width, and 80 MHz repetition rate) and Stokes beams (1032 nm wavelength, 2 ps pulse width, and 80 MHz repetition rate). The Stokes beam was modulated by a built-in electro-optic modulator (EOM) at 20 MHz. The pump and Stokes beams were spatially and temporally overlapped within picoEMERALD. To fill the back aperture of a 25× water objective (XLPLN25XWMP, 1.05 NA, Olympus), the two beams were expanded using a telescope and were guided into an inverted multiphoton laser scanning microscope (FV3000, Olympus). The beams were aligned to maximize the SRS signals of deuterium oxide (99.9 at% D, 151882 Aldrich). The beams transmitted through the sample were collected using a high-NA condenser (oil immersion, 1.4 NA, Olympus). Before detection of the stimulated Raman loss using a large area (10 × 10 mm) Si photodiode (S3590-09, Hamamatsu), the Stokes beam was blocked out using a band-pass filter (893/209 BrightLine, 25 mm, AVR Optics). 64V DC bias voltage was used on the photodiode to increase the saturation threshold and reduce the response time. The output current was terminated by a 50 Ω terminator and prefiltered by a 19.2–23.6 MHz band-pass filter (BBP-21.4+, Mini-Circuits) to reduce laser and scanning noise. A lock-in amplifier (SR844, Stanford Research Systems) demodulated the signals at the modulation frequency. The in-phase X output of the lock-in amplifier was sent to the analog channel of the FV1200 software (FV30-ANALOG). Image acquisition speed was limited by the 30 μs time constant set for the lock-in amplifier. Correspondingly, pixel dwell time is set at 80 μs, yielding a speed of 8.5 s/frame for a 320-by-320-pixel field of view.

SRS spectra were acquired by fixing the Stokes beam at 1031.2 nm and scanning the pump beam through the designated wavelength range for each data point. The total acquisition to record an SRS spectral data cube (40 images) was ~9 min including image acquisition and wavelength tuning. For 1661 cm^{-1} (double bond, ATTO680), 1615 cm^{-1} (double bond, DrBphP-PCM Pfr state), and 1628 cm^{-1} (double bond, DrBphP-PCM Pr state), the wavelengths of the pump laser were 880.4, 884, and 883 nm, respectively. Laser powers on the sample were measured to be 25 mW for the pump beam and 100 mW for the modulated Stokes beam. Laser powers were monitored throughout image acquisition by an internal power meter, and power fluctuations are controlled within 5% by the laser system. Sixteen-bit gray-scale images were acquired by Fluoview software.

Characterization of dye solutions. To characterize photoswitching by transitioning to the first excited state and the triplet state, a solution of 500 μM of Rhodamine 800 dye was prepared without and with 250 mM of potassium iodide (KI), respectively. Figure 1d illustrates the SRS microscopy system used in this study. A 660 nm continuous wave laser (1185057, Coherent), which serves as an excitation beam, was installed and aligned so that it spatially overlapped with the pump and Stokes beam. The alignment precision in all three dimensions was confirmed by measurements on both 1 μm and 200 nm polystyrene beads. The dye molecules were pumped to the excited electronic state by irradiation of 0 mW - 34 mW 660 nm excitation beam on sample concurrently with the pump and Stokes beam. 34 mW power ensures that >80% of the molecules are in the excited state (Table S1).

To test photoswitching by molecular transition to the long-lived dark state, a solution containing 500 μM of ATTO680 and 0.5 M MEA at pH 9.5 was prepared. The pH value was adjusted with 1 M KOH. For absorbance spectra measurement, the solution was loaded into a 100 μL cuvette. The ON state spectrum was measured using a standard spectrophotometer (Cary 500 Scan). To take the OFF state spectrum, the cuvette was illuminated with 100 mW of defocused

660 nm excitation beam for 3 minutes prior to spectrum acquisition. The second ON state spectra was acquired after agitating the same cuvette to facilitate oxidation.

For SRS and fluorescence measurements, the solution was loaded into a glass chamber with a press-to-seal silicone isolator (GBL664501, Sigma) used as a spacer. To measure the epr-SRS/fluorescence signals of the OFF state, the sample solution was illuminated with 100 mW of defocused 660 nm excitation beam for 3-5 seconds prior to SRS/fluorescence measurement. Reactivation was carried out by removing the coverslip, mixing the solution via pipetting up and down, and redepositing the same solution on to a glass slide. The chamber was sealed with a new coverslip for subsequent SRS/fluorescence measurements. For photoswitching experiments shown in Fig. 5b, these depletion and oxidation steps were repeated for multiple cycles on the same solution. To measure the recovery of epr-SRS/fluorescence signals upon irradiation of blue light, the solution sample after depletion was irradiated with focused 405 nm continuous wave laser (Coherent) at 1.1 mW power for ~8 seconds (15 frames at 0.556 s/frame).

Photoswitching characterization of dyes in cells. HeLa cells were seeded on a coverslip in a Petri dish with DMEM for 20 h, which was then replaced with DMEM medium without FBS for another 20 h for synchronization. 10 μM EdU in fresh DMEM medium was then added to cells for 15 h. Cells were fixed with 4% PFA for 20 min and permeabilized with 0.5% Triton for 30 min. 1 μM ATTO680-azide in Click-iT cell reaction buffer (Invitrogen) was then added to cells to react with EdU for 20 min. Cells were washed twice with PBS before imaging. To turn the epr-SRS signals off, the field-of-view was shined with focused 640 nm laser at 2.2 mW power for 80 seconds (200 frames, 0.412 s/frame). To turn the epr-SRS signals back on, the same field-of-view was shined with 405 nm laser at 1.1 mW power for 20 seconds minutes.

Protein expression and characterization. The protein expression and purification followed the standard protocol from previous report.[1] For absorbance spectra measurement, the purified DrBphP-PCM solution was loaded into a 100 μL cuvette. To take the ON state spectrum, the cuvette was illuminated with a defocused 690 nm laser diode (HL6738MG, Thorlabs Inc.) for 1 minute prior to spectrum acquisition using a standard spectrophotometer (Cary 500 Scan). The OFF state spectra was acquired after illuminating the cuvette with a defocused 780 nm near-infrared LED (M780LP1, Thorlabs Inc) for 1 minute. For SRS and fluorescence measurements, the solution was loaded into a glass chamber typically used for SRS measurements. To measure the epr-SRS signal of the OFF state, the sample solution was illuminated with 11 mW of defocused 660 nm continuous wave laser for 1 minute prior to SRS measurement. To measure the epr-SRS/fluorescence signal of the ON state, reactivation was carried out by shining 140 mW of defocused 780 nm light for 1 minute. For photoswitching experiments shown in Fig. 6g & 6h, the activation and deactivation steps were repeated for multiple cycles on the same solution. For fluorescence measurement, 0.11 mW of focused 640 nm light and emission filter with wavelength range 650-750 nm were used. To acquire SRS spectra of DrBphP-PCM at the Pfr state, 1 minute of 780 nm light was irradiated on the protein samples every 7-10 data points to ensure that all the proteins remain in the Pfr state.

$$\sigma_{Raman} = K\omega_{pump}\left(\omega_{pump} - \omega_{vib}\right)^3 \left[\frac{\omega_{pump}^2 + \omega_0^2}{(\omega_0^2 - \omega_{pump}^2)^2}\right]^2$$
$$where\ K \sim {\sigma_{abs}}^2$$

Supplementary Scheme 1. Albrecht A-term pre-resonance approximation. ω_{vib} is the vibrational transition energy. ω_0 is the electronic absorption energy. ω_{pump} is the pump photon energy. *K* is a collection of frequency-independent factors of the Raman dyes. *K* is proportional to the square of absorption cross-section (σ_{abs}).[2–4]

N_0	N_1	Laser power (mW)
50	50	5.6
20	80	22.2
10	90	50
5	95	105.6
1	99	550.5

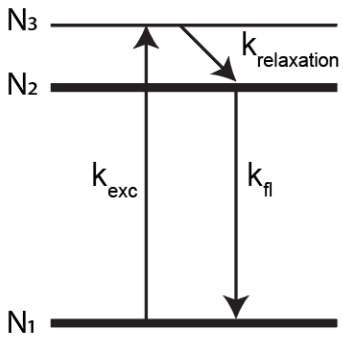

Table S1. Electronic excited state population and required laser power. N_0, ground state population; N_1, excited state population. The molecular transition to the first electronic excited state was modeled as a three-level system. As the vibrational relaxation rate ($k_{relaxation}$) is much faster than the fluorescence emission rate (k_{fl})), the continuous wave excitation at a rate k_{exc} populates the excited state with a steady-state probability $N_1 = k_{exc}/(k_{exc} + k_{fl})$ where $k_{exc} = \sigma I$. k_{fl} is the excited state lifetime, σ is the absorption cross-section at 660 nm, and I is the intensity of the excitation beam. Using k_{fl}= 1/(1.6 ns) and $\sigma = 1.9 \times 10^{-16}$cm^2, I can be solved.

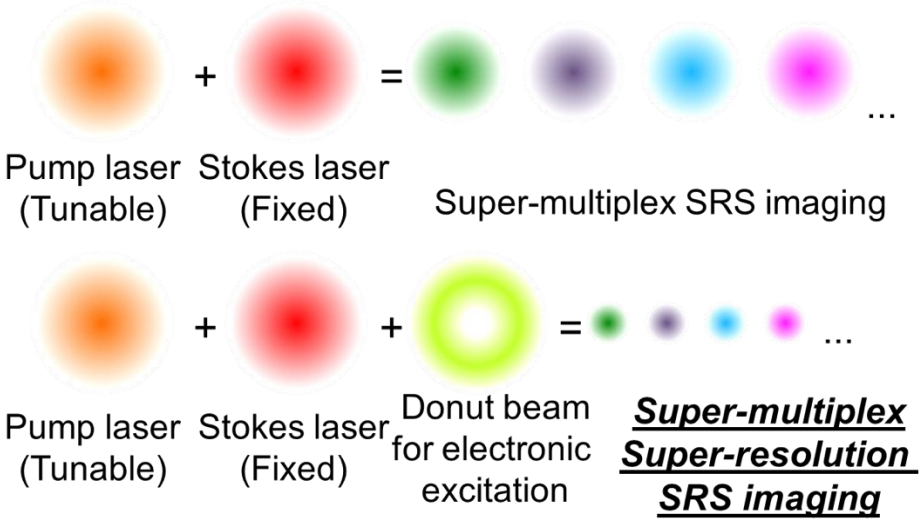

Figure S1. Proposed scheme of super-multiplex super-resolution epr-SRS imaging. Top: scheme for the existing super-multiplex epr-SRS imaging with a fixed Stokes laser (red) and a tunable pump laser (orange). Bottom: scheme for the proposed super-multiplex super-resolution with photoswitchable epr-SRS probes. With one additional doughnut electronic excitation beam (green) to switch the periphery probes to the OFF state and two SRS lasers (orange and red) to image the multiplexable epr-SRS probes in the ON state at the center, effective super-multiplex super-resolution imaging could be envisioned.

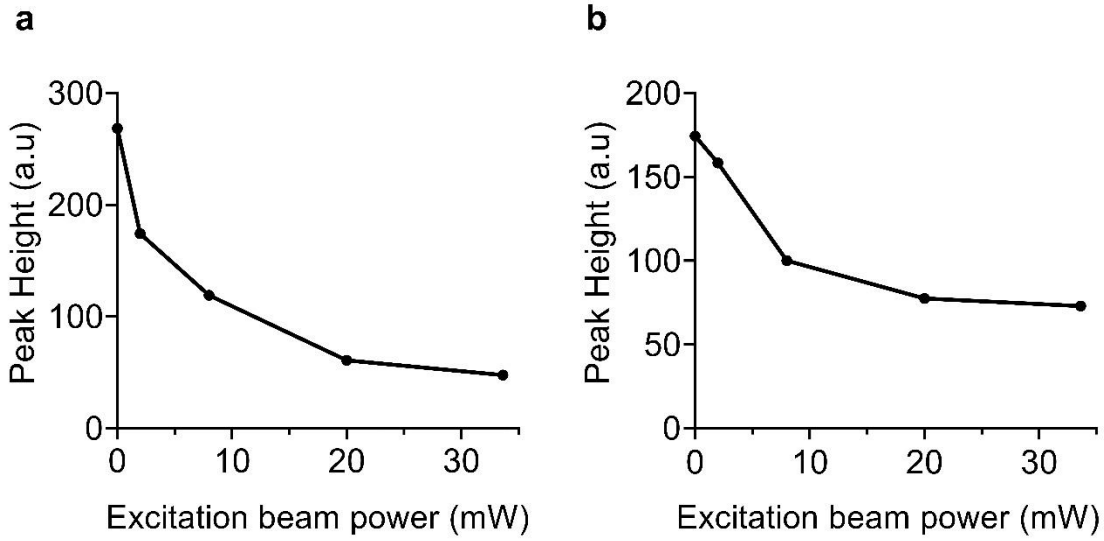

Figure S2. The remaining epr-SRS signals of Rh800 as a function of 660 nm laser powers used to induce transitions to the electronic excited state. (a) Peak height for double-bond signals of Rh800. (b) Peak height for the triple-bond signals of Rh800. Peak heights were calculated by subtracting the epr-SRS intensity at off-resonance frequency (1694.7 cm^{-1} for double bond, 2278.6 cm^{-1} for triple bond) from on-resonance frequency (1655.9 cm^{-1} for double bond, 2235.7 cm^{-1} for triple bond). The original spectral data is shown in Figs. 2b&c.

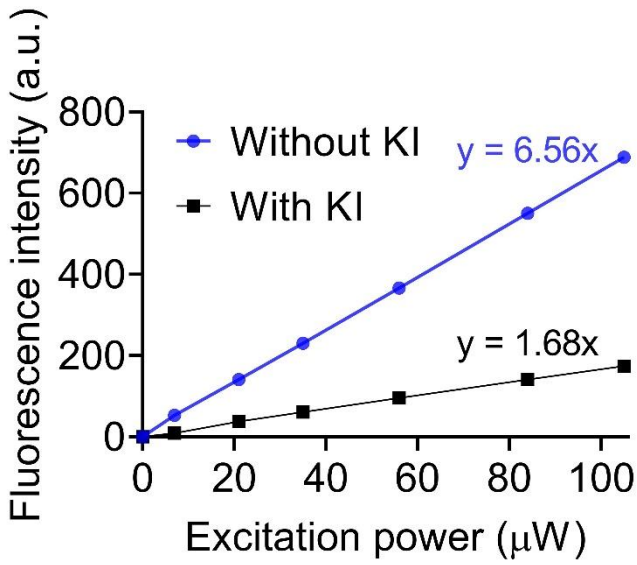

Fig. S3. Comparison of the fluorescence intensities of 500 μM Rh800 solution with (black) and without (blue) KI with 0-100 μW of 640 nm excitation. Although KI is known to induce other transitions including internal conversion from S_1 to S_0 and triplet state quenching by reduction[5–7], k_{ISC} is the most dominant transition at high KI concentration. A factor of 4 decrease in fluorescence intensity with KI indicates an effective shifting of 75% of excited state population to the triplet state compared to that without KI.

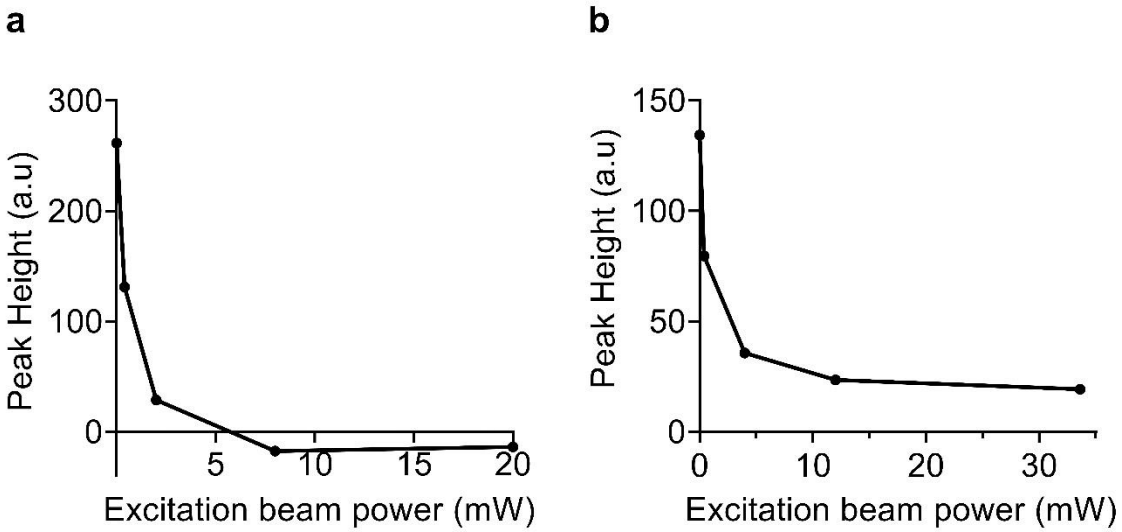

Figure S4. The remaining epr-SRS signals of Rh800 in KI as a function of 660 nm laser powers used to induce transition to the triplet state. (a) Peak height for double-bond signals of Rh800. (b) Peak height was calculated by subtracting the SRS intensity at off-resonance frequency (1694.7 cm^{-1} for double bond, 2278.6 cm^{-1} for triple bond) from on-resonance frequency (1655.9 cm^{-1} for double bond, 2235.7 cm^{-1} for triple bond). The original spectral data is shown in Figs. 3b&c.

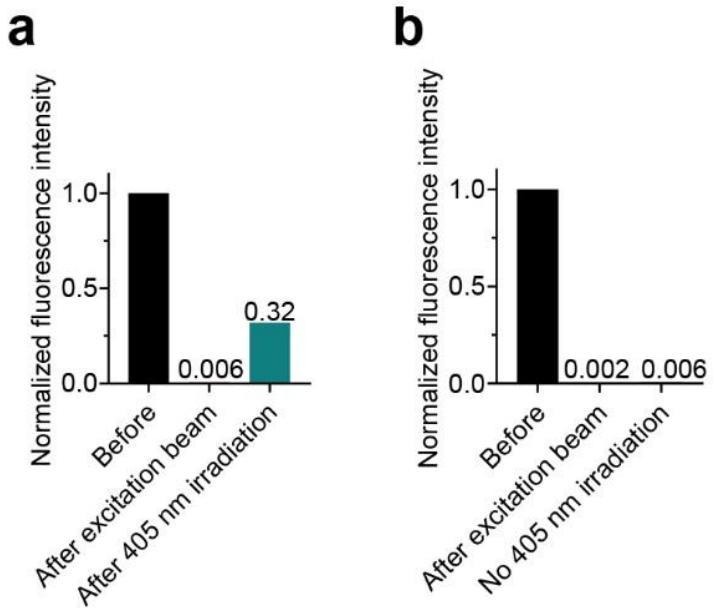

Figure S5. Photoswitching of fluorescence via transition to the long-lived dark state. a) Fluorescence recovery is observed after 405 nm irradiation. b) Photoswitching is not observed in the absence of the 405 nm laser irradiation.

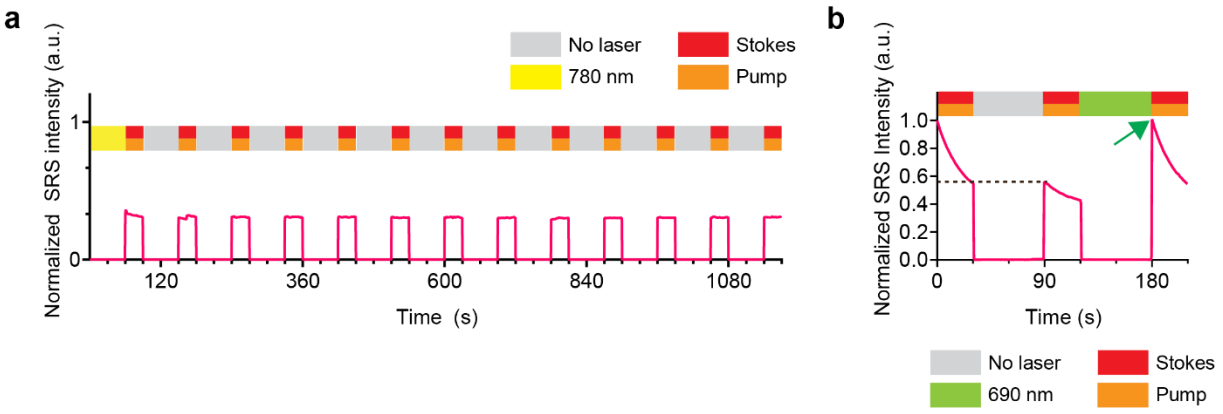

Figure S6. Characterization of photoswitching properties of DrBphP-PCM. a) Photoswitching is not observed in the absence of the activation and deactivation laser for DrBphP-PCM that started in the epr-SRS OFF state. b) Recovery of the epr-SRS signals are only observed when irradiated with 690 nm laser.

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